The Journal of Immunology

Suppression of Osteoprotegerin Expression by Prostaglandin E_2 Is Crucially Involved in Lipopolysaccharide-Induced Osteoclast Formation

Koji Suda, Nobuyuki Udagawa, Nobuaki Sato, Masamichi Takami, Kanami Itoh, Je-Tae Woo, Naoyuki Takahashi and Kazuo Nagai

J Immunol 2004; 172:2504-2510; ;
doi: 10.4049/jimmunol.172.4.2504
http://www.jimmunol.org/content/172/4/2504

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Suppression of Osteoprotegerin Expression by Prostaglandin E_2 Is Crucially Involved in Lipopolysaccharide-Induced Osteoclast Formation[1]

Koji Suda,*† Nobuyuki Udagawa,‡ Nobuaki Sato,‡ Masamichi Takami,† Kanami Itoh,† Je-Tae Woo,¶ Naoyuki Takahashi,[2§] and Kazuo Nagai¶

LPS is a potent stimulator of bone resorption in inflammatory diseases. The mechanism by which LPS induces osteoclastogenesis was studied in cocultures of mouse osteoblasts and bone marrow cells. LPS stimulated osteoclast formation and PGE_2 production in cocultures of mouse osteoblasts and bone marrow cells, and the stimulation was completely inhibited by NS398, a cyclooxygenase-2 inhibitor. Osteoblasts, but not bone marrow cells, produced PGE_2 in response to LPS. LPS-induced osteoclast formation was also inhibited by osteoprotegerin (OPG), a decoy receptor of receptor activator of NF-κB ligand (RANKL), but not by anti-mouse TNFR1 Ab or IL-1 receptor antagonist. LPS induced both stimulation of RANKL mRNA expression and inhibition of OPG mRNA expression in osteoblasts. NS398 blocked LPS-induced down-regulation of OPG mRNA expression, but not LPS-induced up-regulation of RANKL mRNA expression, suggesting that down-regulation of OPG expression by PGE_2 is involved in LPS-induced osteoclast formation in the cocultures. NS398 failed to inhibit LPS-induced osteoclastogenesis in cocultures containing OPG knockout mouse-derived osteoblasts. IL-1 also stimulated PGE_2 production in osteoblasts and osteoclast formation in the cocultures, and the stimulation was inhibited by NS398. As seen with LPS, NS398 failed to inhibit IL-1-induced osteoclast formation in cocultures with OPG-deficient osteoblasts. These results suggest that IL-1 as well as LPS stimulates osteoclastogenesis through two parallel events: direct enhancement of RANKL expression and suppression of OPG expression, which is mediated by PGE_2 production. ***The Journal of Immunology,*** **2004, 172: 2504–2510.**

Osteoclasts are bone-resorbing multinucleated cells that originate from hemopoietic progenitors of the monocyte/macrophage lineage (1–4). Osteoblasts or bone marrow stromal cells are involved in osteoclastogenesis through a mechanism involving cell-to-cell contact with osteoclast progenitors (4, 5). Studies of M-CSF-deficient *op/op* mice have shown that M-CSF produced by osteoblasts is an essential factor for osteoclastogenesis (6, 7). Receptor activator of NF-κB ligand (RANKL)[3] (3) was also identified as another factor essential for osteoclastogenesis (8–11). RANKL is a member of the TNF-ligand family that is expressed by osteoblasts/stromal cells as a membrane-associated factor. Osteoclast precursors express RANK, a receptor of RANKL; recognize RANKL through cell-cell interaction; and differentiate into osteoclasts in the presence of M-CSF (12, 13). Osteoblasts/Stromal cells also produce a soluble decoy receptor for RANKL, osteoprotegerin (OPG), which inhibits osteoclast formation in vivo and in vitro by interrupting the interaction between RANKL and RANK (14, 15).

In mouse cell cocultures, osteoclasts are formed in response to bone-resorbing factors such as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), parathyroid hormone (PTH), PGE_2, and IL-11 (4). Almost all of the bone-resorbing factors stimulate expression of RANKL in osteoblasts/stromal cells (4). Three independent signals have been proposed to induce RANKL expression in osteoblasts/stromal cells: vitamin D receptor-mediated signals induced by $1,25(OH)_2D_3$, cAMP/protein kinase A (PKA)-mediated signals induced by PTH or PGE_2, and gp130-mediated signals induced by IL-11 (4). Among these signals, vitamin D receptor- and cAMP/PKA-mediated signals suppress OPG expression in osteoblasts/stromal cells. Recently, we reported that compounds that elevate intracellular calcium, such as ionomycin, A23187, cyclopiazonic acid, and thapsigargin, stimulated osteoclast formation in mouse cocultures (16). Treatment of primary osteoblasts with those compounds stimulated the expression of RANKL. Thus, the signal mediated by calcium and protein kinase C (PKC) is proposed to be another (fourth) signal that induces RANKL expression in osteoblasts/stromal cells.

Severe bone loss due to excessive bone resorption is observed in inflammatory diseases such as periodontitis and osteomyelitis and some types of arthritides (17). LPS, a major constituent of Gram-negative bacteria, is proposed to be a potent stimulator of bone loss in these inflammatory diseases (17–22). Recently, Toll-like receptor 4 (TLR4) was identified as the signal-transducing receptor for LPS (23, 24). The cytoplasmic signaling cascade of TLR4 is similar to that of IL-1Rs. Both TLR4 and IL-1Rs use common signaling molecules such as myeloid differentiation factor 88 and TNFR-associated factor 6 (25–29). Macrophages, lymphocytes,

*Department of Bioengineering, Tokyo Institute of Technology, Yokohama, Japan; †Department of Biochemistry, School of Dentistry, Showa University, Tokyo, Japan; ‡Department of Biochemistry, School of Dentistry, and §Institute for Oral Science, Matsumoto Dental University, Shiojiri, Japan; and ¶Department of Biological Chemistry, Chubu University, Kasugai, Japan

Received for publication August 26, 2003. Accepted for publication November 18, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported in part by grants-in-aid (12137209, 13557155, 13470394, and 14207075) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. K.S. is Research Fellow of the Japan Society for the Promotion of Science.

[2] Address correspondence and reprint requests to Dr. Naoyuki Takahashi, Institute for Oral Science, Matsumoto Dental University, 1780 Gobara, Hiro-oka, Shiojiri-shi, Nagano 399-0781, Japan. E-mail address: takahashinao@po.mdu.ac.jp

[3] Abbreviations used in this paper: RANKL, receptor activator of NF-κB ligand; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; COX2, cyclooxygenase 2; EIA, enzyme immunoassay; ERK, extracellular signal-regulated kinase; IL-1ra, IL-1 receptor antagonist; OPG, osteoprotegerin; PKA, protein kinase A; PKC, protein kinase C; PTH, parathyroid hormone; RANK, receptor activator of NF-κB; TLR, Toll-like receptor; TRAP, tartrate-resistant acid phosphatase.

0022-1767/04/$02.00

and osteoblasts/stromal cells express TLR4, and produce PGE_2 and proinflammatory cytokines such as TNF-α and IL-1 in response to LPS (30, 31). These inflammatory factors also stimulate osteoclastogenesis directly or indirectly (17–22, 32).

LPS stimulates PGE_2 production in the target cells through the induction of mitogen-inducible cyclooxygenase 2 (COX2) expression (33). NS398, a nonsteroidal anti-inflammatory agent, specifically inhibits COX2 without affecting COX1 activity (34). Therefore, NS398 has been used as a tool to explore the role of PGE_2 in pathological processes involving COX2 activity. PGE_2 exerts its biological actions through binding to four specific membrane receptors (EP1, EP2, EP3, and EP4) (35). Sakuma et al. (20, 21) reported that induction of osteoclast formation by LPS, TNF-α, and IL-1 was barely observed in cell cultures prepared from EP4 knockout ($EP4^{-/-}$) mice, and that urinary excretion of deoxypyridinoline, a sensitive marker for bone resorption, was not increased in $EP4^{-/-}$ mice injected with LPS. These results suggest that PGE_2 is a key factor in the enhancement of osteoclastogenesis by LPS in vivo and in vitro. However, it is still not known how PGE_2 is involved in the induction of osteoclastogenesis by LPS.

In the present study, we examined the mechanism of the induction of osteoclast formation by LPS in cocultures of mouse osteoblasts and bone marrow cells. We showed that LPS promoted osteoclastogenesis through two parallel events: one was direct enhancement of RANKL expression, and the other was suppression of OPG production mediated by PGE_2 in osteoblasts. In addition, IL-1 stimulated osteoclast formation in the cocultures in a manner similar to LPS.

Materials and Methods

Reagents and mice

LPS (*Escherichia coli* O26:B6) and PGE_2 were purchased from Sigma-Aldrich (St. Louis, MO). NS398 was from Calbiochem (San Diego, CA). Human rOPG and mouse rIL-1β were obtained from PeproTech (London, U.K.). Mouse rTNF-α and mouse rIL-1 receptor antagonist (rIL-1ra) were obtained from R&D Systems (Minneapolis, MN). Anti-mouse TNFR1 Ab was obtained from Genzyme Diagnostics (Cambridge, MA). Six- to 9-wk-old male and newborn ddY mice were obtained from Sankyo Laboratory Animal Center (Tokyo, Japan). C57BL/6 (B6) mice and OPG-deficient ($OPG^{-/-}$) mice (C57BL/6 (B6)) were obtained from Clear Japan Clea (Tokyo, Japan). This study was reviewed and approved by the Showa University Animal Care and Use Committee.

Cell preparation and osteoclast formation assay

Primary osteoblasts were obtained from calvariae of newborn ddY mice, C57BL/6 (B6) mice, and $OPG^{-/-}$ mice by the conventional method using collagenase (36). Bone marrow cells were collected from femora and tibiae of 6- to 9-wk-old male mice. Primary osteoblasts (1×10^4 cells) and bone marrow cells (2×10^5 cells) were cocultured for 5 days in α-MEM containing 10% FCS (CSL, Victoria, Australia) in 96-well tissue culture plates (Corning, Corning, NY) (0.2 ml/well). Cocultures were incubated in the presence of LPS (0.001–10 μg/ml), PGE_2 (1 μM), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) for the final 3 days. Some cocultures were pretreated with NS398 (1 μM), OPG (100 ng/ml), IL-1ra (10 μg/ml), and TNFR1 Ab (10 μg/ml) for 1 h before adding LPS, PGE_2, IL-1β, or TNF-α. Then cocultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts). TRAP-positive cells containing >3 nuclei were counted as osteoclasts. The results obtained from a typical experiment of three independent experiments are expressed as the mean ± SD of four cultures.

Measurement of PGE_2 production

Primary osteoblasts (3×10^4 cells) and bone marrow cells (6×10^5 cells) were cultured separately or in combination with or without LPS (1 μg/ml) or IL-1β (10 ng/ml) in α-MEM containing 10% FCS in 48-well culture plates (Corning). After the cultures were incubated for 6 h, the concentration of PGE_2 in the culture medium was determined using an enzyme immunoassay (EIA; Cayman Chemicals, Ann Arbor, MI). The Ab showed the following cross-reactivity determined by comparing the bond/free ratios with several eicosanoids: PGE_2, 100%; PGE_2 ethanolamide, 100%; PGE_3, 43%; PGE_1, 18.7%; 6-keto $PGF_1\alpha$, 1%; and 8-*iso* $PGF_2\alpha$, 0.25%.

Northern blot analysis

Primary osteoblasts (1×10^6 cells) were seeded in cell culture dishes (60 mm in diameter; Corning) and cultured in α-MEM containing 10% FCS for 3 days. After incubation in α-MEM containing 0.1% FCS for 3 h, the cells were incubated with LPS (1 μg/ml) or IL-1β (10 ng/ml). In some experiments, osteoblasts were cocultured with bone marrow cells (2×10^7 cells) in the presence of LPS (1 μg/ml) for 3 or 48 h. Some cultures were also treated with NS398 (1 μM) for 1 h before the addition of LPS. Total RNA was isolated from cultures using TRIzol (Life Technologies, Grand Island, NY). Northern blot analysis was performed using denaturing formaldehyde/agarose gels, as described (16). Double-stranded cDNA fragments encoding mouse RANKL OPG and COX2 were kindly provided by H. Yasuda (Snow Brand Milk Products, Tochigi, Japan). cDNA probes (RANKL, OPG, COX2, and β-tubulin) labeled with ^{32}P were synthesized using a cDNA labeling kit (Takara, Tokyo, Japan). The RANKL, OPG, COX2, and β-tubulin probes were hybridized with membranes to which total RNA isolated from osteoblasts had been transferred. The membranes were exposed to Kodak BioMax MS film (Rochester, NY) for 3–48 h. Signals of RANKL, OPG, COX2, and β-tubulin mRNA were quantified using a radioactive image analyzer (BAS2000; Fuji Photo Film, Tokyo, Japan). Signals of RANKL, OPG, and COX2 mRNAs were normalized with the respective β-tubulin mRNA expression levels to calculate the relative intensity.

Results

PGE_2 is required for osteoclast formation induced by LPS

LPS stimulated TRAP-positive osteoclast formation in cocultures of primary osteoblasts and bone marrow cells in a dose-dependent manner (Fig. 1*A*). The maximal number of osteoclasts was observed at 1 μg/ml of LPS. We then examined whether PGE_2 is involved in the induction of osteoclast formation by LPS. LPS (1 μg/ml) as well as PGE_2 (1 μM) induced TRAP-positive osteoclast formation in the cocultures (Fig. 1, *B* and *C*). NS398 (1 μM), a specific inhibitor of COX2, suppressed the induction of osteoclast formation by LPS, but not by PGE_2 in the cocultures (Fig. 1, *B* and *C*). Both LPS- and PGE_2-induced osteoclast formation in the cocultures was strongly inhibited by simultaneous addition of OPG (100 ng/ml) (Fig. 1, *B* and *C*). These results suggest that both PGE_2 production and RANKL-RANK interaction are required for LPS-induced osteoclast formation in the cocultures. We next examined whether LPS induces osteoclastogenesis through IL-1 and TNF-α. Recombinant IL-1β (10 ng/ml) and TNF-α (10 ng/ml) induced osteoclastogenesis in the cocultures. IL-1ra (10 μg/ml) and anti-mouse TNFR1 Ab (10 μg/ml) strongly inhibited the osteoclast formation induced by IL-1β and TNF-α, respectively. However, neither IL-1ra nor TNFR1 Ab affected osteoclastogenesis induced by LPS (Fig. 1*D*). These results suggest that PGE_2 is a critical factor in LPS-induced osteoclastogenesis.

Osteoblasts mainly produce PGE_2 in response to LPS

We then measured the PGE_2 concentration in the conditioned medium of cocultures incubated with or without LPS (1 μg/ml) for 6 h (Fig. 2*A*). LPS significantly increased the PGE_2 concentration in the culture medium (Fig. 2*A*). The addition of NS398 (1 μM) to the cocultures completely blocked the induction of PGE_2 production by LPS in the cocultures (Fig. 2*A*). To determine the type of cells that respond to LPS in the cocultures, osteoblasts and bone marrow cells were cultured separately in the presence or absence of LPS for 6 h (Fig. 2*B*). LPS stimulated PGE_2 production in the cultures of osteoblasts, but not bone marrow cells. LPS-induced PGE_2 production was strongly inhibited by the addition of NS398 (Fig. 2*B*). Northern blot analysis showed that treatment of osteoblasts with LPS for 3 h stimulated the expression of COX2 mRNA (Fig. 2*C*). These results suggest that osteoblasts in the cocultures

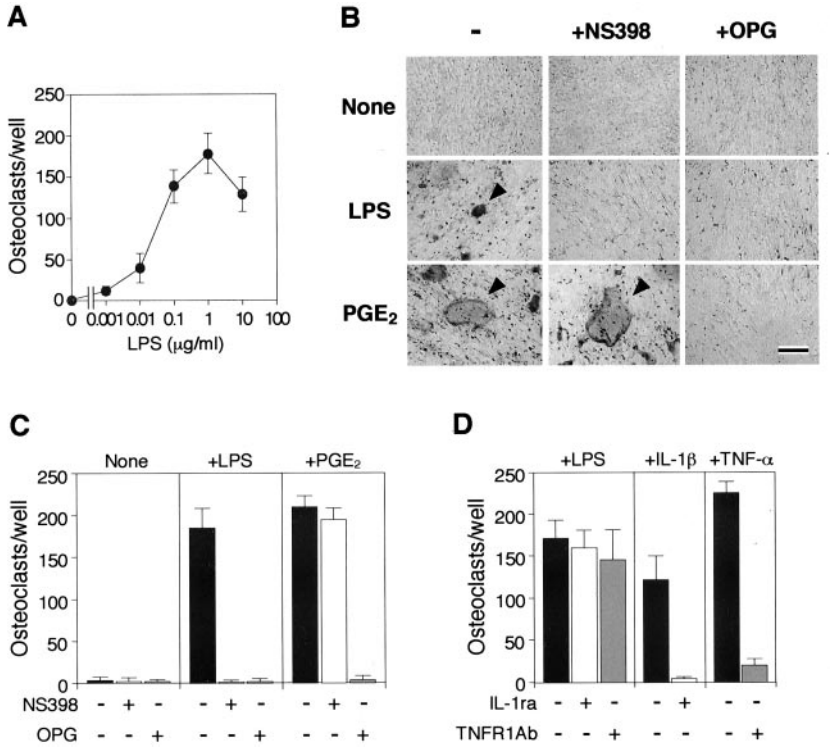

FIGURE 1. Effects of NS398, OPG, IL-1ra, and TNFR1 Ab on osteoclast formation in cocultures treated with LPS. *A*, Mouse primary osteoblasts and bone marrow cells were cocultured for 5 days. LPS (0.001–10 μg/ml) was added to the cocultures for the final 3 days. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *B*, LPS (1 μg/ml) or PGE_2 (1 μM) together with or without NS398 (1 μM) or OPG (100 ng/ml) was added to the cocultures for the final 3 days. The cells were then fixed and stained for TRAP. Arrowheads indicate TRAP-positive multinucleated cells. Bar, 200 μm. *C*, TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *D*, LPS (1 μg/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) together with or without IL-1ra (10 μg/ml) and TNFR1 Ab (10 μg/ml) was added to the cocultures for the final 3 days. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

produce PGE_2 in response to LPS via up-regulation of COX2 mRNA expression.

LPS regulates RANKL and OPG gene expression in osteoblasts

We next analyzed the effects of LPS on RANKL and OPG mRNA expression levels in primary osteoblasts by Northern blot analysis (Fig. 3). Treatment of the osteoblasts with LPS increased RANKL mRNA expression with two peaks at 3 and 48 h. The expression of RANKL mRNA after treatment with LPS was increased within 1 h, and was still higher than that of the control cultures even after 72 h (Fig. 3*A*). The expression of OPG mRNA in osteoblasts was also enhanced by the treatment with LPS for 3 h (Fig. 3*A*). However, the expression of OPG mRNA in osteoblasts treated with LPS for 48 or 72 h was decreased to a level lower than that of the control culture (Fig. 3*A*). NS398 (1 μM) had no effect on the level of RANKL mRNA induced by LPS at 3 h (Fig. 3*B*). The LPS-induced up-regulation of RANKL mRNA expression at 48 h was slightly inhibited by the COX2 inhibitor, but the level of the mRNA was much higher than that in the control cultures. In contrast, the LPS-induced down-regulation of OPG mRNA expression in osteoblasts at 48 h after treatment with LPS was completely blocked by the addition of NS398, although the OPG mRNA expression at 3 h was not affected by the COX2 inhibitor (Fig. 3*B*). The expression levels of RANKL and OPG mRNAs in bone marrow cells were lower than those in primary osteoblasts, and were unchanged even after treatment with NS398 for 3 or 48 h (data not shown). These results suggest that PGE_2 produced by osteoblasts plays an important role in the down-regulation of OPG expression, but not the up-regulation of RANKL expression in osteoblasts treated with LPS.

Suppression of OPG expression is involved in induction of osteoclast formation by LPS

We next examined how PGE_2 production is involved in LPS-induced osteoclast formation using osteoblasts from OPG-deficient ($OPG^{-/-}$) mice. Primary osteoblasts prepared from $OPG^{-/-}$ mice were cocultured with bone marrow cells from wild-type mice in the presence or absence of NS398, OPG, and/or LPS (Fig. 4). In agreement with previously reported findings (37), TRAP-positive osteoclasts were formed in cocultures containing $OPG^{-/-}$ osteoblasts even in the absence of any stimulus (Fig. 4). The number of osteoclasts was further increased in the LPS-treated cocultures containing $OPG^{-/-}$ osteoblasts. NS398 strongly suppressed the spontaneous osteoclast formation in the control cocultures containing $OPG^{-/-}$ osteoblasts. This suggests that endogenous production of PGE_2 plays an important role in the osteoclast formation in cocultures containing $OPG^{-/-}$ osteoblasts. NS398 slightly, but not completely, inhibited LPS-induced osteoclast formation in cocultures containing $OPG^{-/-}$ osteoblasts (Fig. 4). OPG completely suppressed osteoclast formation in the cocultures treated or not treated with LPS (Fig. 4). These results suggest that the down-regulation of OPG expression by PGE_2 is crucially involved in the osteoclast formation induced by LPS in the cocultures.

IL-1 stimulates osteoclast formation in a manner similar to LPS

Because the signaling pathway of IL-1Rs is quite similar to that of TLR4 (26–30), we finally examined whether IL-1 stimulates osteoclastogenesis in the cocultures in a manner similar to LPS. IL-1β (10 ng/ml) induced osteoclast formation in the wild-type cocultures, and the induction was inhibited by NS398 (1 μM) and OPG (100 ng/ml) (Fig. 5*A*). IL-1β (10 ng/ml) also stimulated PGE_2 production in osteoblasts, but not in bone marrow cells after treatment for 6 h (Fig. 5*B*). Northern blot analysis showed that IL-1β up-regulated COX2 mRNA expression in osteoblasts at 3 h (Fig. 5*C*). IL-1β also stimulated RANKL mRNA expression at 3 h (data not shown). IL-1β stimulated osteoclast formation in the cocultures of $OPG^{-/-}$ osteoblasts and wild-type bone marrow cells (the control: 53 ± 11, the mean ± SD of four cultures) (Fig. 5*D*). NS398 (1 μM) did not completely suppress LPS-induced osteoclast formation in the cocultures with $OPG^{-/-}$ osteoblasts, but

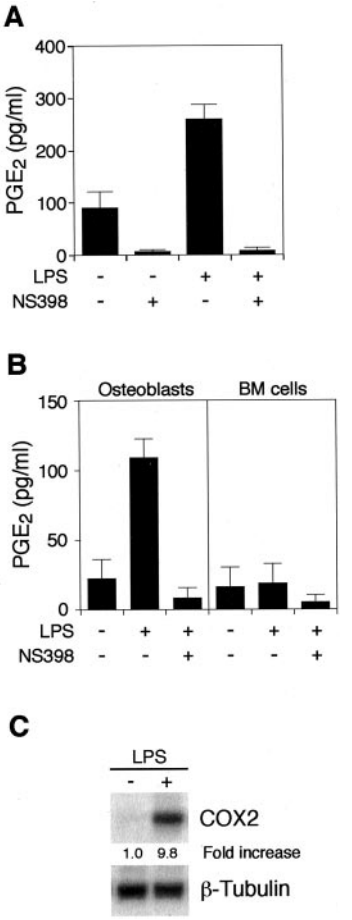

FIGURE 2. LPS induces PGE_2 production and COX2 expression in osteoblasts. *A*, Primary osteoblasts and bone marrow (BM) cells were cocultured with LPS (1 μg/ml) in the presence or absence of NS398 (1 μM). After the cultures were incubated for 6 h, the concentration of PGE_2 in the culture supernatant was determined using EIA. Values are expressed as the means $\pm$ SD of quadruplicate cultures. *B*, Primary osteoblasts and bone marrow cells were cultured separately with LPS (1 μg/ml) in the presence or absence of NS398 (1 μM) for 6 h. The PGE_2 concentration in the culture supernatant was determined using EIA. Values are expressed as the means $\pm$ SD of quadruplicate cultures. *C*, Primary osteoblasts were treated with LPS (1 μg/ml) for 3 h. Total RNA was isolated from the osteoblasts, and COX2 and β-tubulin mRNA expression was analyzed by Northern blotting. Figures below the signals represent the intensity of the COX2 mRNA signals relative to the β-tubulin mRNA signals.

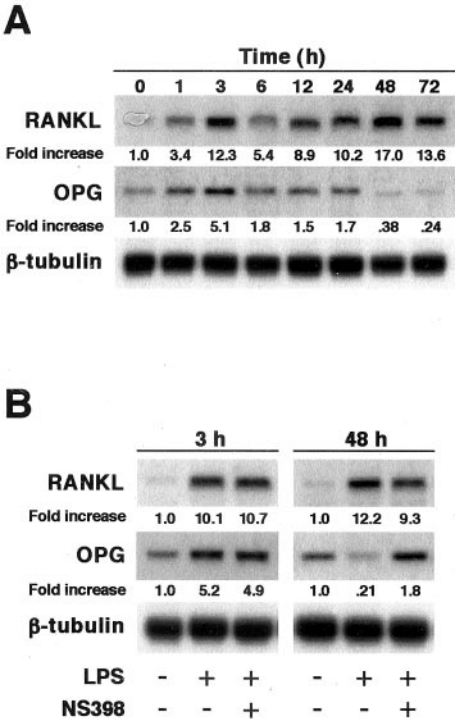

FIGURE 3. LPS regulates the expression of RANKL and OPG mRNAs in osteoblasts. *A*, Primary osteoblasts were treated with LPS (1 μg/ml) for 0–72 h. Total RNA was isolated from the osteoblasts, and the expression of RANKL, OPG, and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of the RANKL and OPG mRNA signals relative to the β-tubulin mRNA signals. *B*, Primary osteoblasts were treated with LPS (1 μg/ml) for 3 or 48 h in the presence of bone marrow cells. NS398 (1 μM) was also added to some cultures. After incubation for the indicated periods, bone marrow cells were removed by pipetting. Total RNA was isolated from osteoblasts, and the expression of RANKL, OPG, and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of the RANKL and OPG mRNA signals relative to the β-tubulin mRNA signals.

OPG did (Fig. 5*D*). These results suggest that IL-1 and LPS stimulate osteoclast formation in the same manner in the cocultures.

Discussion

In vivo and in vitro experiments have shown that PGE_2 is crucially involved in the induction of osteoclastic bone resorption by IL-1, TNF-α, and LPS (20). EP4 subtype-mediated signaling has been shown to be particularly important for the induction of bone resorption by such inflammation-related factors as well as PGE_2 (20). The present study showed that LPS stimulated COX2 expression and PGE_2 production in osteoblasts, and NS398, a specific inhibitor of COX2, strongly blocked the LPS-induced osteoclast formation in cocultures containing wild-type osteoblasts (Figs. 1 and 2). These results suggest that PGE_2 is somehow involved in LPS-induced osteoclast formation in the cocultures through PGE_2 receptors of EP4 subtype.

It was reported that LPS stimulated the expression of RANKL mRNA in osteoblasts obtained from $EP4^{-/-}$ mice, and that COX inhibitors did not block this stimulation (21). In agreement with this finding, NS398 failed to inhibit the induction of RANKL expression by LPS in osteoblasts (Fig. 3). These results suggest that LPS induced RANKL expression in a manner that was independent of PGE_2 production in osteoblasts. In contrast, the treatment of osteoblasts in the cocultures with LPS together with NS398 blocked the down-regulation of OPG mRNA expression at 48 h (Fig. 3). This suggests that suppression of OPG by PGE_2 is an important event in osteoclast formation in the cocultures treated with LPS. This notion was further supported by the finding that LPS stimulated osteoclast formation even in the presence of NS398 in cocultures containing osteoblasts derived from $OPG^{-/-}$ mice (Fig. 4). Thus, PGE_2 appears to play an important role as a suppressor of OPG expression rather than an activator of RANKL expression in LPS-induced osteoclast formation (Fig. 6). Recently, Fu et al. (38) reported that the activation of CREB by PTH is required for PTH-induced down-regulation of OPG expression. This suggests that the cAMP-PKA signals play a role in PGE_2-induced suppression of OPG mRNA expression. Further study will elucidate the detail mechanism of the down-regulation of OPG expression by PGE_2.

PGE_2 has been shown to induce RANKL mRNA expression in osteoblasts (10). Suzawa et al. (39) reported that PGE_2-induced RANKL expression is mediated through the cAMP signaling pathway. In our experiments, NS398 failed to inhibit RANKL expression in osteoblasts treated with LPS for as long as 48 h (Fig. 3).

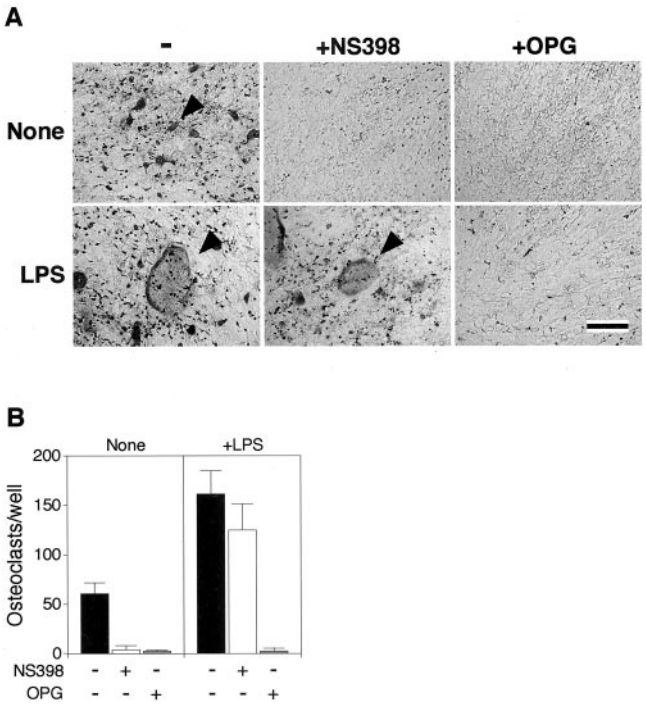

FIGURE 4. Effects of NS398 on LPS-induced osteoclast formation in cocultures containing $OPG^{-/-}$ mouse-derived osteoblasts. *A*, Primary osteoblasts prepared from $OPG^{-/-}$ mice were cocultured with wild-type bone marrow cells for 5 days. LPS (1 μg/ml) was added to the cocultures with or without NS398 (1 μM) or OPG (100 ng/ml) for the final 3 days. The cells were then fixed and stained for TRAP. Arrowheads indicate the TRAP-positive osteoclasts. Bar, 200 μm. *B*, TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

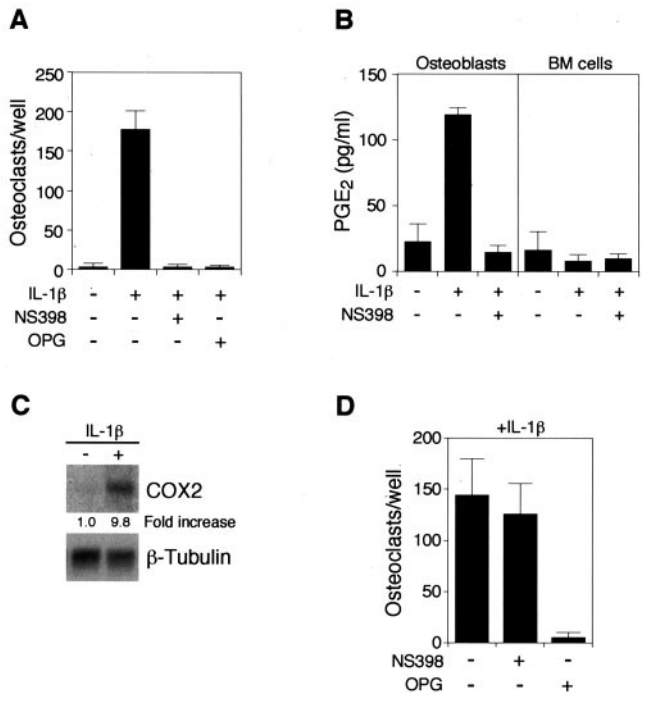

FIGURE 5. IL-1 induces osteoclast formation in the cocultures in a manner similar to LPS. *A*, Mouse primary osteoblasts and bone marrow (BM) cells were cocultured with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) or OPG (100 ng/ml). TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *B*, Primary osteoblasts and bone marrow cells were cultured separately with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) for 6 h. The PGE_2 concentration in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. *C*, Primary osteoblasts were treated with IL-1β (10 ng/ml) for 3 h, and then COX2 and β-tubulin mRNA expression was analyzed by Northern blotting. Figures below the signals represent the intensity of the COX2 mRNA signals relative to the β-tubulin mRNA signals. *D*, Primary osteoblasts prepared from $OPG^{-/-}$ mice and wild-type bone marrow cells were cocultured with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) or OPG (100 ng/ml). TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

This suggests that LPS induces RANKL expression by the mechanism independent of PGE_2 production. Kikuchi et al. (40) reported that LPS induces RANKL through extracellular signal-regulated kinase (ERK) and PKC. We also confirmed that calcium/PKC inhibitors, such as BAPTA-AM (an intracellular calcium chelator) and Ro-32-0432 (a PKC inhibitor), and ERK inhibitor PD98059 inhibited LPS-induced RANKL mRNA expression in osteoblasts (K.S., unpublished observation). PD98059 failed to inhibit the induction of RANKL mRNA expression by PGE_2 and the induction of osteoclast formation in cocultures treated with PGE_2 (data not shown). These results suggest that LPS directly stimulates RANKL expression through calcium/PKC signals, followed by ERK signals in osteoblasts. It is unlikely that PGE_2-induced signals directly cross talk with LPS-induced signals in the induction of RANKL expression in osteoblasts.

The intracellular signaling pathway of TLR4 is quite similar to that of IL-1Rs (25–29). Like LPS, IL-1 stimulated COX2 mRNA expression at 3 h and PGE_2 production at 6 h in osteoblast cultures (Fig. 5). IL-1β also induced RANKL mRNA expression in osteoblasts, as previously reported (data not shown) (32). NS398 inhibited IL-1-induced osteoclast formation strongly in cocultures containing wild-type osteoblasts, but only partially in cocultures containing $OPG^{-/-}$ osteoblasts (Fig. 5). These results suggest that PGE_2 produced by osteoblasts in response to IL-1 plays a similar role to LPS in osteoclast formation through the suppression of OPG expression (Fig. 6).

In cocultures containing $OPG^{-/-}$ osteoblasts, osteoclasts were formed even in the absence of any stimulus (Fig. 4). The spontaneous osteoclast formation was strongly inhibited by the addition of either OPG or NS398 (Fig. 4). These results suggest that RANKL is involved in the spontaneous osteoclast formation, and that PGE_2 constitutively produced in the cocultures stimulates RANKL expression in osteoblasts. LPS and IL-1β further enhanced osteoclast formation in cocultures containing $OPG^{-/-}$ osteoblasts (Fig. 4), suggesting that the up-regulation of RANKL expression by LPS and IL-1 enhances the osteoclast formation. The induction of osteoclast formation by LPS and IL-1 in cocultures containing $OPG^{-/-}$ osteoblasts was partially inhibited by the addition of NS398. Therefore, PGE_2 induced by LPS and IL-1 appears to be involved in RANKL expression in osteoblasts. Our results indicate that the full inhibition of LPS- and IL-1-induced osteoclast formation by NS398 requires PGE_2-dependent suppression of OPG production (Fig. 6).

The previous studies have shown that OPG production by osteoblasts is down-regulated by bone-resorbing factors such as $1,25(OH)_2D_3$, PTH, and PGE_2 (38, 41–44). Our results confirmed the previous finding that osteoclasts spontaneously form in the control cocultures containing $OPG^{-/-}$ osteoblasts. The decrease in OPG production by osteoblasts was a key event for the induction

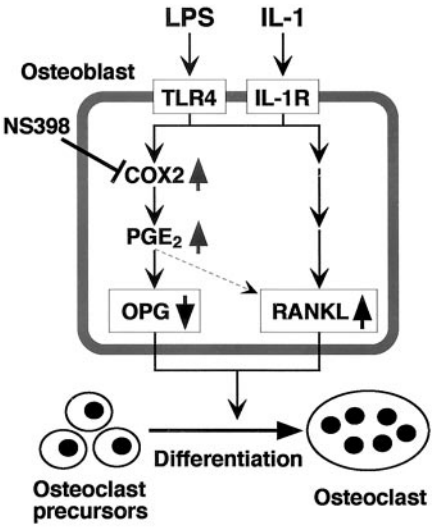

FIGURE 6. A possible mechanism of the induction of osteoclastogenesis by LPS and IL-1. LPS and IL-1 promote the differentiation of osteoclast precursors into osteoclasts through two parallel events in osteoblasts: direct enhancement of RANKL expression, and suppression of OPG production mediated by PGE_2. PGE_2 induced by LPS and IL-1 also stimulates RANKL expression, but the suppression of OPG production in osteoblasts appears to be more important than the induction of RANKL expression in osteoblasts for the stimulation of osteoclastogenesis. See text for details.

of osteoclastogenesis by LPS and IL-1. $OPG^{-/-}$ mice exhibited severe osteoporosis caused by enhanced osteoclast formation and function (14, 15, 45, 46). These results suggest that OPG is a physiological regulator of bone resorption, and that the balance between RANKL and OPG expressions at bone is particularly important for the regulation of bone resorption in vivo and in vitro.

We previously reported that LPS and IL-1 directly stimulated the survival, fusion, and pit-forming activity of osteoclasts (47). Those results together with the results shown in this study suggest that LPS and IL-1 are involved in the stimulation of osteoclastic bone resorption in several ways: LPS and IL-1 directly stimulate osteoclast function, induce RANKL expression in osteoblasts, and suppress OPG expression through enhancement of PGE_2 production. Further studies will be necessary to elucidate the precise mechanism of the regulation of osteoclastic bone resorption induced by these inflammatory factors.

References

1. Roodman, G. D. 1999. Cell biology of the osteoclast. *Exp. Hematol. 27:1229.*
2. Chambers, T. J. 2000. Regulation of the differentiation and function of osteoclasts. *J. Pathol. 192:4.*
3. Teitelbaum, S. L. 2000. Bone resorption by osteoclasts. *Science 289:1504.*
4. Suda, T., N. Takahashi, N. Udagawa, E. Jimi, M. T. Gillespie, and T. J. Martin. 1999. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr. Rev. 20:345.*
5. Takahashi, N., T. Akatsu, N. Udagawa, T. Sasaki, A. Yamaguchi, J. M. Moseley, T. J. Martin, and T. Suda. 1988. Osteoblastic cells are involved in osteoclast formation. *Endocrinology 123:2600.*
6. Yoshida, H., S. Hayashi, T. Kunisada, M. Ogawa, S. Nishikawa, H. Okamura, T. Sudo, L. D. Shultz, and S. Nishikawa. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature 345:442.*
7. Felix, R., M. G. Cecchini, and H. Fleisch. 1990. Macrophage colony stimulating factor restores in vivo bone resorption in the *op/op* osteopetrotic mouse. *Endocrinology 127:2592.*
8. Wong, B. R., J. Rho, J. Arron, E. Robinson, J. Orlinick, M. Chao, S. Kalachikov, E. Cayani, F. S. Bartlett III, W. N. Frankel, et al. 1997. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J. Biol. Chem. 272:25190.*
9. Anderson, D. M., E. Maraskovsky, W. L. Billingsley, W. C. Dougall, M. E. Tometsko, E. R. Roux, M. C. Teepe, R. F. DuBose, D. Cosman, and L. Galibert. 1997. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature 390:175.*
10. Yasuda, H., N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, et al. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. USA 95:3597.*
11. Lacey, D. L., E. Timms, H. L. Tan, M. J. Kelley, C. R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, et al. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell 93:165.*
12. Jimi, E., S. Akiyama, T. Tsurukai, N. Okahashi, K. Kobayashi, N. Udagawa, T. Nishihara, N. Takahashi, and T. Suda. 1999. Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. *J. Immunol. 163:434.*
13. Li, J., I. Sarosi, X. Q. Yan, S. Morony, C. Capparelli, H. L. Tan, S. McCabe, R. Elliott, S. Scully, G. Van, et al. 2000. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc. Natl. Acad. Sci. USA 97:1566.*
14. Shimonet, W. S., D. L. Lacey, C. R. Dunstan, M. Kelley, M. S. Chang, R. Luthy, H. Q. Nguyen, S. Wooden, L. Bennett, T. Boone, et al. 1997. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell 89:309.*
15. Tsuda, E., M. Goto, S. Mochizuki, K. Yano, F. Kobayashi, T. Morinaga, and K. Higashio. 1997. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem. Biophys. Res. Commun. 234:137.*
16. Takami, M., N. Takahashi, N. Udagawa, C. Miyaura, K. Suda, J. T. Woo, T. J. Martin, K. Nagai, and T. Suda, 2000. Intracellular calcium and protein kinase C mediate expression of receptor activator of nuclear factor-κB ligand and osteoprotegerin in osteoblasts. *Endocrinology 141:4711.*
17. Nair, S. P., S. Meghji, M. Wilson, K. Reddi, P. White, and B. Henderson. 1996. Bacterially induced bone destruction: mechanisms and misconceptions. *Infect. Immun. 64:2371.*
18. Abu-Amer, Y., F. P. Ross, J. Edwards, and S. L. Teitelbaum. 1997. Lipopolysaccharide-stimulated osteoclastogenesis is mediated by tumor necrosis factor via its p55 receptor. *J. Clin. Invest. 100:1557.*
19. Ito, H. O., T. Shuto, H. Takada, T. Koga, Y. Aida, and M. Hirata. 1996. Lipopolysaccharides from *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* promote osteoclastic differentiation in vitro. *Arch. Oral Biol. 41:439.*
20. Sakuma, Y., K. Tanaka, M. Suda, A. Yasoda, K. Natsui, I. Tanaka, F. Ushikubi, S. Narumiya, E. Segi, Y. Sugimoto, et al. 2000. Crucial involvement of the EP4 subtype of prostaglandin E receptor in osteoclast formation by proinflammatory cytokines and lipopolysaccharide. *J. Bone Miner. Res. 15:218.*
21. Sakuma, Y., K. Tanaka, M. Suda, Y. Komatsu, A. Yasoda, M. Miura, A. Ozasa, S. Narumiya, Y. Sugimoto, A. Ichikawa, et al. 2000. Impaired bone resorption by lipopolysaccharide in vivo in mice deficient in the prostaglandin E receptor EP4 subtype. *Infect. Immun. 68:6819.*
22. Ueda, N., M. Koide, M. Ohguchi, Y. Ishihara, T. Noguchi, N. Okahashi, and T. Nishihara. 1998. Involvement of prostaglandin E_2 and interleukin-1α in the differentiation and survival of osteoclasts induced by lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4. *J. Periodontal Res. 33:509.*
23. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science 282:2085.*
24. Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med. 189:615.*
25. Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity 9:145.*
26. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity 11:115.*
27. Romaga, M. A., W. C. Yeh, I. Sarosi, G. S. Duncan, C. Furlonger, A. Ho, S. Morony, C. Capparelli, G. Van, S. Kaufman, et al. 1999. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. *Genes Dev. 13:1015.*
28. Naito, A., S. Azuma, S. Tanaka, T. Miyazaki, S. Takaki, K. Takatsu, K. Nakao, K. Nakamura, M. Katsuki, T. Yamamoto, and J. Inoue. 1999. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes Cells 4:353.*
29. Zhang, F. X., C. J. Kirschning, R. Mancinelli, X. P. Xu, Y. Jin, E. Faure, A. Mantovani, M. Rothe, M. Muzio, and M. Arditi. 1999. Bacterial lipopolysaccharide activates nuclear factor-κB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J. Biol. Chem. 274:7611.*
30. Lynn, W. A., and D. T. Golenbock. 1992. Lipopolysaccharide antagonists. *Immunol. Today 13:271.*
31. Schletter, J., H. Heine, A. J. Ulmer, and E. T. Rietschel. 1995. Molecular mechanisms of endotoxin activity. *Arch. Microbiol. 164:383.*
32. Hofbauer, L. C., D. L. Lacey, C. R. Dunstan, T. C. Spelsberg, B. L. Riggs, and S. Khosla. 1999. Interleukin-1β and tumor necrosis factor-α, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone 25:255.*
33. Hwang, D. 2001. Modulation of the expression of cyclooxygenase-2 by fatty acids mediated through Toll-like receptor 4-derived signaling pathways. *FASEB J. 15:2556.*
34. Futaki, N., S. Takahashi, M. Yokoyama, I. Arai, S. Higuchi, and S. Otomo. 1994. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. *Prostaglandins 47:55.*

35. Coleman, R. A., W. L. Smith, and S. Narumiya. 1994. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev. 46:205*.
36. Suda, T., E. Jimi, I. Nakamura, and N. Takahashi. 1997. Role of 1α, 25-dihydroxyvitamin D_3 in osteoclast differentiation and function. *Methods Enzymol. 282:223*.
37. Udagawa, N., N. Takahashi, H. Yasuda, A. Mizuno, K. Itoh, Y. Ueno, T. Shinki, M. T. Gillespie, T. J. Martin, K. Higashio, and T. Suda. 2000. Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. *Endocrinology 141:3478*.
38. Fu, Q., R. L. Jilka, S. C. Manolagas, and C. A. O'Brien. 2002. Parathyroid hormone stimulates receptor activator of NF-κB ligand and inhibits osteoprotegerin expression via protein kinase A activation of cAMP-response element-binding protein. *J. Biol. Chem. 277:48868*.
39. Suzawa, T., C. Miyaura, M. Inada, T Maruyama, Y Sugimoto, F. Ushikubi, A. Ichikawa, S. Narumiya, and T. Suda. 2000. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology 141:1554*.
40. Kikuchi, T., T. Matsuguchi, N. Tsuboi, A. Mitani, S. Tanaka, M. Matsuoka, G. Yamamoto, T. Hishikawa, T. Noguchi, and Y. Yoshikai. 2001. Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors. *J. Immunol. 166:3574*.
41. Brandstrom, H., K. B. Jonsson, C. Ohlsson, O. Vidal, S. Ljunghall, and O. Ljunggren. 1998. Regulation of osteoprotegerin mRNA levels by prostaglandin E_2 in human bone marrow stroma cells. *Biochem. Biophys. Res. Commun. 247:338*.
42. Horwood, N. J., J. Elliott, T. J. Martin, and M. T. Gillespie. 1998. Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. *Endocrinology 139:4743*.
43. Lee, S. K., and J. A. Lorenzo. 1999. Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation. *Endocrinology 140:3552*.
44. Onyia, J. E., R. R. Miles, X. Yang, D. L. Halladay, J. Hale, A. Glasebrook, D. McClure, G. Seno, L. Churgay, S. Chandrasekhar, and T. J. Martin. 2000. In vivo demonstration that human parathyroid hormone 1-38 inhibits the expression of osteoprotegerin in bone with the kinetics of an immediate early gene. *J. Bone Miner. Res. 15:863*.
45. Mizuno, A., N. Amizuka, K. Irie, A. Murakami, N. Fujise, T. Kanno, Y. Sato, N. N akagawa, H. Yasuda, S. Mochizuki, et al. 1998. Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. *Biochem. Biophys. Res. Commun. 247:610*.
46. Bucay, N., I. Sarosi, C. R. Dunstan, S. Morony, J. Tarpley, C. Capparelli, S. Scully, H. L. Tan, W. Xu, D. L. Lacey, et al. 1998. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev. 12:1260*.
47. Suda, K., J. T. Woo, M. Takami, P. M. Sexton, and K. Nagai. 2000. Lipopolysaccharide supports survival and fusion of preosteoclasts independent of TNF-α, IL-1, and RANKL. *J. Cell. Physiol. 190:101*.